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Thimerosal Neurotoxicity is Associated with Glutathione Depletion: Protection with Glutathione Precursors

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Abstract

Thimerosol is an antiseptic containing 49.5% ethyl mercury that has been used for years as a preservative in many infant vaccines and in flu vaccines. Environmental methyl mercury has been shown to be highly neurotoxic, especially to the developing brain. Because mercury has a high affinity for thiol (sulfhydryl (–SH)) groups, the thiol-containing antioxidant, glutathione (GSH), provides the major intracellular defense against mercury-induced neurotoxicity. Cultured neuroblastoma cells were found to have lower levels of GSH and increased sensitivity to thimerosol toxicity compared to glioblastoma cells that have higher basal levels of intracellular GSH. Thimerosal-induced cytotoxicity was associated with depletion of intracellular GSH in both cell lines. Pretreatment with 100 μM glutathione ethyl ester or N-acetylcysteine (NAC), but not methionine, resulted in a significant increase in intracellular GSH in both cell types. Further, pretreatment of the cells with glutathione ethyl ester or NAC prevented cytotoxicity with exposure to 15 μM Thimerosal. Although Thimerosal has been recently removed from most children's vaccines, it is still present in flu vaccines given to pregnant women, the elderly, and to children in developing countries. The potential protective effect of GSH and cysteine against mercury toxicity warrants further research as possible adjunct therapy to individuals still receiving Thimerosal-containing vaccinations.

Keywords: Thimerosal; Neurotoxicity; Glutathione depletion

INTRODUCTION

Thimerosal (sodium ethylmercurithiosalicylate) was developed by Eli Lilly in the 1930s as a effective bacteriostatic and fungistatic preservative and has been widely used in multidose vials of vaccines and in ophthalmic, otic, nasal, and topical products. Until the removal of Thimerosal from most pediatric vaccines in 2001, the largest human exposure in the US (μg/kg body weight) was in children under 18 months of age undergoing routine childhood immunization schedules. Prior to 2001, a child may have received a cumulative dose of over 200 μg/kg in the first 18 months of life (Ball et al., 2001). Although the neurotoxicity of methyl mercury has been relatively well studied, limited information is available on the relative neurodevelopmental toxicity of ethylmercury, the mercury metabolite of Thimerosal. Based on the known toxicity of methylmercury, the cumulative ethylmercury exposure to US pediatric populations in Thimerosal-containing vaccinations was re-examined in 1999 and found to exceed EPA recommended guidelines

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(Ball et al., 2001). Following recommendations by the American Academy of Pediatrics and the US Public Health Service (Public Health Service, 1999), Thimerosal was subsequently removed as a preservative from most children's vaccines in the US. However, influenza vaccines and Rho D immunoglobulin shots containing Thimerosal are still recommended to pregnant women, and many vaccines given to children in developing countries still contain Thimerosal. The present study was undertaken to better understand the mechanisms underlying Thimerosal toxicity to neurons and astrocytes, the primary CNS targets for organic mercury (Sanfeliu et al., 2001). A better understanding of the neurotoxic mechanism is a necessary prerequisite for the design of intervention strategies for prevention and for the identification of genetic variants that could increase sensitivity to Thimerosal.

Previous mechanistic studies of methylmercury toxicity in astrocytes and neurons have implicated reactive oxygen species (ROS) and depletion of intracellular glutathione as major contributors to mercury-induced cytotoxicity (Sanfeliu et al., 2001). Organic mercury has a high affinity for the thiol (–SH) group on glutathione, a tripeptide composed of cysteine, glutamate, and glycine (Sanfeliu et al., 2003). The cysteine moiety of glutathione carries the active thiol group that binds and detoxifies a variety of heavy metals, including organic and inorganic mercury. Normally, the intracellular concentration of glutathione is extremely high, in the mM range (Meister, 1995); however, with depletion of this essential antioxidant, excess free mercury is available to bind to cysteine thiol groups present in essential cellular proteins, leading to functional inactivation and cytotoxicity.

The synthesis of glutathione in the brain is unique in that brain cells do not express cystathionine gamma lyase, an enzyme in the transsulfuration pathway involved in glutathione synthesis (Awata et al., 1995). As a result, brain cells cannot synthesize cysteine, the rate limiting amino acid for glutathione synthesis. Thus, the brain is dependent on the liver to synthesize and export cysteine for uptake and utilization by astrocytes and neurons for adequate glutathione synthesis (Lu, 1998). In contrast to the brain, the liver expresses the complete transsulfuration pathway from methionine to cysteine and glutathione as diagrammed in Fig. 1. Glutathione synthesized in the liver is exported to the plasma where it is immediately degraded to cysteinylglycine and cysteine (Lu, 1998). Cysteine is converted to cystine in the oxidizing environment of the plasma and subsequently transported to the brain for intracellular glutathione synthesis (Fig. 2).

A second unusual aspect of glutathione synthesis in the brain is the unique metabolic interaction between astrocytes and neurons regarding uptake of cysteine, the rate-limiting amino acid for glutathione synthesis (Dringen and Hirrlinger, 2003; Kranich et al., 1996). Astrocytes and neurons have different affinities for the uptake of oxidized and reduced forms of cysteine for glutathione synthesis (Dringen et al., 2000b). Neurons are unable to take up cystine (oxidized plasma form of cysteine) but can readily transport reduced cysteine for glutathione synthesis. In contrast, oxidized cystine is readily taken up by astrocytes and converted to glutathione as diagrammed in Fig. 2 (Kranich et al., 1998; Wang and Cynader, 2000). Because cysteine is the rate-limiting amino acid for glutathione synthesis, the relative availability

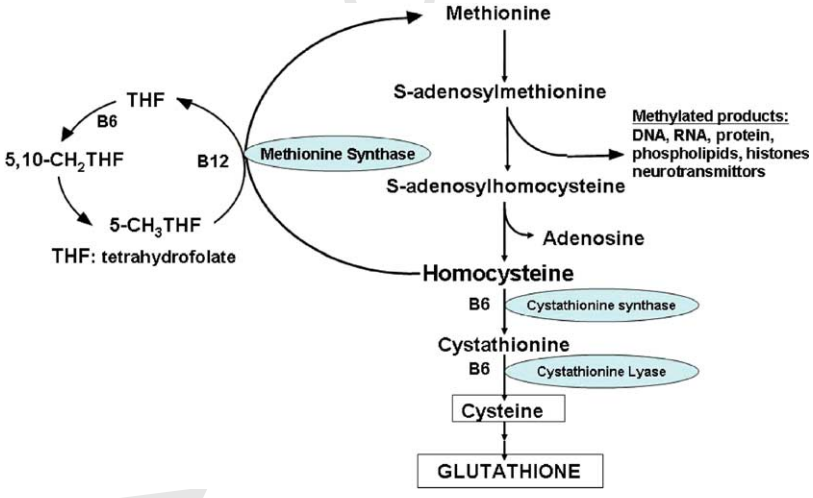

Fig. 1. Pathways of glutathione synthesis. The liver expresses the complete pathway from methionine through homocysteine and cysteine to glutathione. Astrocytes and neurons do not express the enzyme cystathionine lyase and therefore are unable to synthesize cysteine. As a result, astrocytes and neurons are dependent on plasma cysteine derived primarily from the liver to synthesize glutathione.

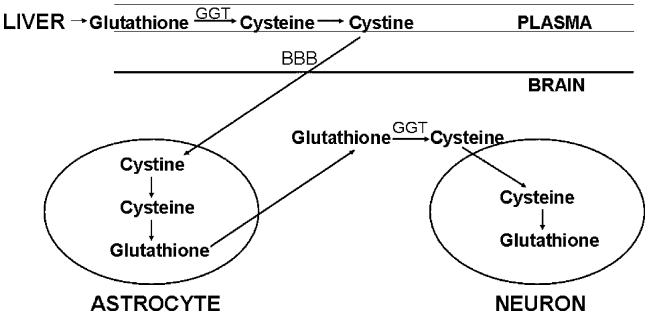

Fig. 2. Interaction between astrocytes and neurons for glutathione synthesis. Glutathione exported from the liver is hydrolyzed to cysteinylglycine and cysteine. In the plasma, cysteine is oxidized to cystine and transported across the BBB and taken up by astrocytes and used to synthesize glutathione. Astrocytes export glutathione into the extracellular space where it is hydrolyzed to cysteine and taken up by neurons for glutathione synthesis. Abbreviations: GGT: gamma glutamyl synthetase; BBB: blood–brain barrier.

of extracellular cystine and cysteine determines intracellular glutathione concentrations and resistance to mercury toxicity in astrocytes and neurons, respectively.

The purpose of the present study was to determine the relative sensitivity of astrocytes and neurons to Thimerosal (ethyl mercury) cytotoxicity in vitro and to determine whether Thimerosal neurotoxicity was associated with depletion of glutathione in cultured human cells as previously reported for methylmercury (Sanfeliu et al., 2001). Acute high dose exposures to Thimerosal (μmol/L) in cultured cells were used to study mechanistic aspects of Thimerosal toxicity and not intended to mimic exposures of developing brain cells in vivo to Thimerosal in vaccines (nmol/kg).

MATERIALS AND METHODS

Materials

Culture flasks, 96-well plates, and pipettes were obtained from Falcon (Franklin Lakes, NJ, USA). F-12K and MEM culture media were purchased from ATCC (Manassas, VA, USA) and the RPMI 1640 culture media, streptomycin, and Dulbecco's Phosphate Buffered Saline were purchased from Gibco (Grand Island, NY, USA). The TACS™ MTT kit for cell viability assay was purchased from R & D systems (Minneapolis, MN, USA). Fetal bovine serum was purchased from HyClone (Logan, UT, USA). Thimerosal USP, glutathione ethyl ester, cysteine, cystine, *N*-acetyl-L-cysteine, L-methionine, and EDTA-trypsin were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell Culture

Human neuroblastoma SH-SY5Y CRL 2266 cells and glioblastoma CRL 2020 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured in T-25 ml flasks in a humidified incubator at 37 °C with 5% CO_2. The neuroblastoma cells were cultured in 50:50 F-12K media and MEM media containing 15% fetal calf serum and 1% penicillin/streptomycin. The glioblastoma cells were cultured in RPMI 1640 media with supplements recommended for this cell line by ATCC plus 15% fetal calf serum with 1% penicillin/streptomycin.

Cell Viability Assays

Cell viability before and after Thimerosal exposure was assessed using the MTT assay. Metabolically active mitochondrial dehydrogenases convert the tetrazolium salt, MTT, to insoluble purple formazan crystals at a rate that is proportional to cell viability. For the viability study with Thimerosal exposure, the cultured neuroblastoma and glioblastoma cells were harvested with 0.25% trypsin and replated in 96-well microtitre plates at a concentration of 6.5×10^5 cells/ml and 5×10^5 cells/ml, respectively, in a 100 μl volume. After a 3-day culture period to reach confluency, 10 μl of Thimerosal in PBS was added to achieve final concentrations of 2.5, 5, 10 or 20 μmol/L in triplicate wells. Pilot studies conducted with increasing duration of exposure at 37 °C indicated that a 48 h incubation period was the threshold for toxicity in the glioblastoma cells whereas only a 3 h incubation was required to achieve similar cytotoxicity in the neuroblastoma cells (data not shown). At the end of the respective incubation periods, 10 μl MTT solution (1:10 v/v) to each well for 1 h followed by 100 μl of dimethylsulfoxide detergent solution to lyse the cells and solubilize the formazan crystals formed in the metabolically active (viable) cells. Triplicate untreated negative control cells were run together with the Thimerosal-treated cells. The optical density (OD) was read in the 96-well plate using the Thermo Max spectrophotometer (Molecular Devices, Sunnyvale, CA) set at 550 nm (with a reference wavelength of 650 nm). Triplicate wells with reagent only served as background controls. The results are expressed as the OD after background subtraction.

Nutrient Supplementation Studies

For the viability studies, cells were exposed to 15 μmol/L Thimerosal with and without prior incubation with *N*-acetylcysteine, cystine, glutathione ethyl ester, or methionine at a final concentration of 100 μmol/L each. *N*-Acetylcysteine is an acetylated analog of cysteine that easily crosses the cell membrane and is rapidly deacetylated inside the cell and utilized for GSH synthesis (Zafarullah et al., 2003). Glutathione ethyl ester is an esterified form of glutathione that is able to cross the cell membrane against the concentration gradient (Anderson et al., 2004). Cystine is the disulfide (oxidized) form of cysteine that is readily taken up by astrocytes, but not neurons (Sagara et al., 1993). Since astrocytes are unable to synthesize GSH from methionine, the addition of methionine to the media served as a negative control. Triplicate aliquots of neuroblastoma and astroglial cells were plated in 96-well plates at concentrations of 8×10^5 cells/ml and 5×10^5 cells/ml, respectively. The supplements were added to the culture media 45 min before the addition of Thimerosal and were prepared as 10x concentrates and added in 10 μL to the cell culture media. Cell viability after Thimerosal exposure was assessed with the MTT assay as described above. For HPLC analysis of intracellular glutathione levels, neuroblastoma and glioblastoma cells were plated in triplicate in 6-well plates at a density of 6.5×10^5 cells/well and 1×10^6 cells/well, respectively, and cultured for 4 days in order to generate sufficient cells for HPLC analysis. Triplicate wells were pooled for analysis.

HPLC Sample Preparation

Briefly, 10^6 cells were homogenized on ice in 200 μL of phosphate-buffered saline. To reduce sulfhydryl (thiol) bonds, 50 μl freshly prepared 1.43 M sodium borohydride solution containing 1.5 μM EDTA, 66 mM NaOH and 10 μl iso-amyl alcohol was added to the homogenate. After mixing, the solution was incubated in a 40 °C water bath for 30 min with gentle shaking. To precipitate proteins, 250 μL ice cold 10% *meta*-phosphoric acid was added, mixed well, and the sample was incubated for 30 min on ice. After centrifugation at 18,000 × *g* for 15 min at 4 °C, the supernatant was filtered through a 0.2 μm nylon membrane filter (PGC Scientific, Frederic, MD). A 20 μl aliquot of cell extract was directly injected onto the column using Beckman Autosampler (model 507E).

HPLC with Coulometric Electrochemical Detection

The elution of glutathione was accomplished using HPLC with a Shimadzu solvent delivery system (ESA model 580) and a reverse phase C18 column (5 μm; 4.6 mm × 150 mm, MCM Inc., Tokyo, Japan) obtained from ESA Inc. (Chemsford, MA). A 20 μL aliquot of cell extract was directly injected onto the column using Beckman autosampler (model 507E). The mobile phase consisted of 50 mM sodium phosphate monobasic, monohydrate, 1.0 mM ion-pairing reagent octane sulfonic acid, 2% (v/v) acetonitrile adjusted to pH 2.7 with 85% phosphoric acid, with isocratic elution at ambient temperature at a flow rate of 1.0 ml/min and a pressure of 120–140 kgf/cm^2 (1800–2100 psi). To assure standardization between sample runs, calibration standards and reference plasma samples were interspersed at intervals during each run. The metabolites were quantified using a model 5200A Coulochem II electrochemical detector (ESA Inc.) equipped with a dual analytical cell (model 5010) and a guard cell (model 5020). The concentration of intracellular glutathione was calculated from peak areas and standard calibration curves using HPLC software and expressed per mg protein. An aliquot of initial homogenate was used for protein determination using the BCA protein assay (Pierce Inc., Rockford, IL).

Statistics

Values are expressed as the mean ± standard deviation. One way ANOVA was used to determine significant differences between groups and the Bonferroni *t*-test for pairwise comparisons using Sigma Stat 2.0 software. Treatment related differences were considered to be significant at $p < 0.05$.

RESULTS

Dose–Response Characteristics of Thimerosal in Glioblastoma and Neuroblastoma Cells

Fig. 3A and B shows the dose response characteristics of triplicate cultures of the glioblastoma and neuroblastoma cells, respectively. In both cell lines, a progressive increase in cytotoxicity (decrease in viability) was observed when Thimerosal dose was progressively doubled from 2.5 μmol/L to 5, 10 and 20 μmol/L. Viability was reduced more than 50% in

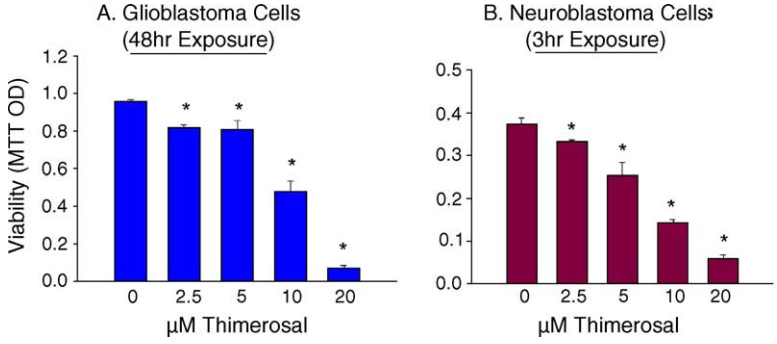

Fig. 3. Viability of glioblastoma cells (A) and neuroblastoma cells (B) with increasing concentrations of Thimerosal in the media. Asterisks indicate significant differences from control cells without Thimerosal treatment ($n = 3$, $p < 0.01$).

both cell lines with exposure to 10 μmol/L Thimerosal and less than 10% of cells survived a dose of 20 μmol/L. Although the shape of the dose response is similar between the two cell types, the dose response curve for the neuroblastoma cells occurred with a only a 3 h exposure whereas the glioblastoma cell required 48 h to exhibit similar cytotoxic effects at the same dose. These results demonstrate that the neuroblastoma cells are much more sensitive to Thimerosal cytoxicity than are glioblastoma cells.

Neuroprotective Effect of *N*-Acetylcysteine, Cystine, and Glutathione Ethyl Ester

Based on the viability assays, 15 μmol/L Thimerosal was selected as the dose for the supplementation studies since a significant level of toxicity was observed in both cell lines.

Glioblastoma Cells

Triplicate cultures were pretreated for 1 h with 100 μmol/L *N*-acetylcysteine, glutathione ethyl ester, cystine, or methionine before addition of 15 μmol/L Thimerosal to the culture medium. As shown in Fig. 4A, 15 μmol/L Thimerosal alone induced approximately 3-fold decrease in cell viability whereas pretreatment with either cystine, glutathione, or NAC provided significant protection from cell death. The failure of methionine to provide precursors for GSH synthesis is consistent with the lack of cystathionine γ lyase activity in these cells.

Neuroblastoma Cells

Triplicate cultures of neuroblastoma cells were pretreated for 1 h with the same supplements as the glioblastoma cells. Fig. 4B demonstrates that Thimerosal alone induced more than a 6-fold decrease in viability, confirming the increased vulnerability of neuroblastoma cells to Thimerosal relative to the glioblastoma cells. Both NAC and glutathione ethyl ester provided significant protection against cell death whereas cystine and methionine were without effect. The lack of effect of cystine is consistent with previous reports that neurons can take up cysteine, but not cystine (Sagara et al., 1993). As expected, methionine afforded no protection.

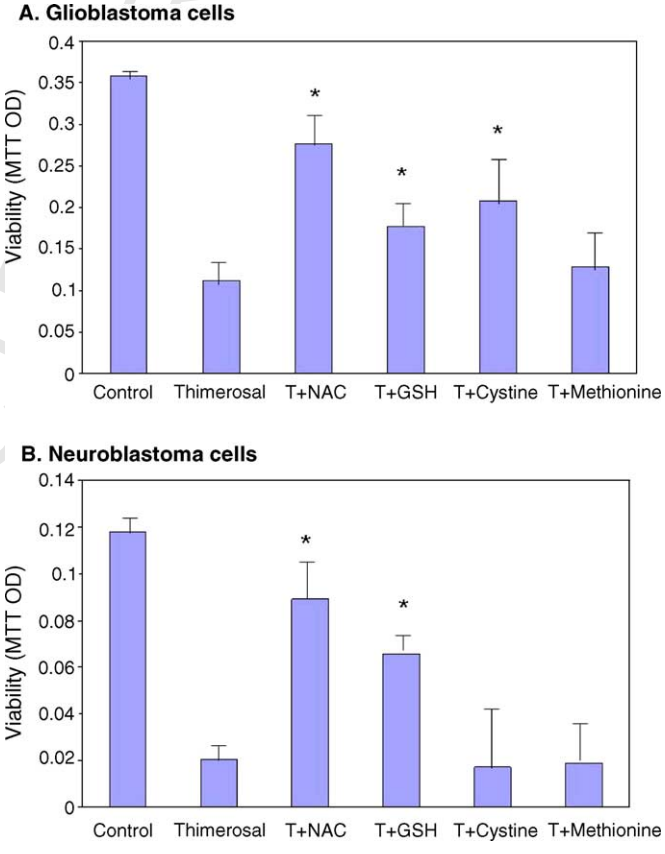

Fig. 4. Viability of glioblastoma cells (A) and neuroblastoma cells (B) without (control) and with exposure to 15 μmol/L Thimerosal alone for 48 h or Thimerosal treatment after a 45 min pretreatment with 100 μmol/L of *N*-acetyl cysteine (NAC), glutathione ethyl ester (GSH) cystine, or methionine. Asterisks indicate significant differences between cells treated with Thimerosal alone and cells pretreated with indicated nutrients ($n = 3$, $p < 0.05$).

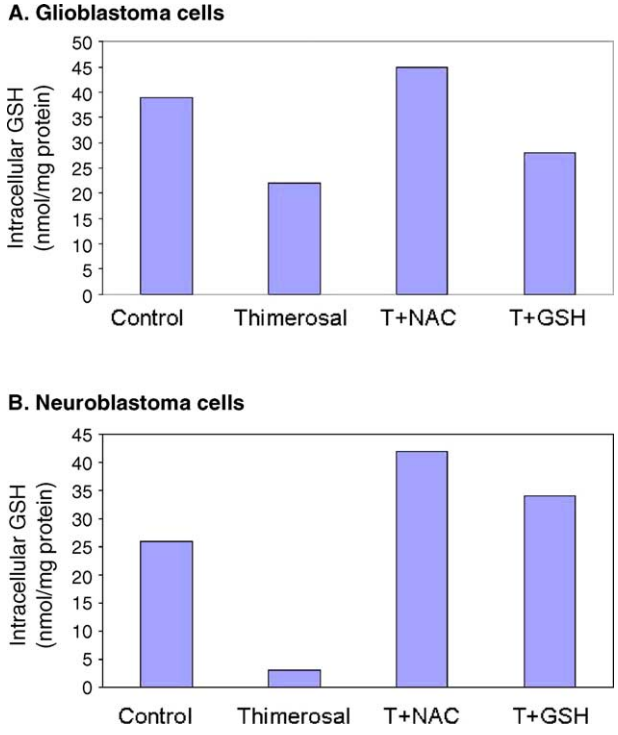

Fig. 5. Mean concentration of intracellular glutathione in 10^6 glioblastoma cells (A) and neuroblastoma cells (B) without (control) and with 15 μmol/L Thimerosal alone or Thimerosal after pretreatment with 100 μmol/L of *N*-acetyl cysteine (NAC), glutathione ethyl ester (GSH) in a representative experiment that was repeated with similar results.

Glutathione Depletion with Thimerosal Exposure: Preservation of Intracellular GSH with Nutritional Supplementation

The baseline level of intracellular glutathione was 39 μmol/L in the glioblastoma cells compared to only 26 μmol/L in the neuroblastoma cells (Fig. 5A). Exposure to15 μmol/L Thimerosal for 1 h caused less than a 50% decrease in intracellular glutathione levels in the glioblastoma cells whereas the same exposure induced more than an 8-fold-decrease in the neuroblastoma cells. Pretreatment with NAC or glutathione ethyl ester completely prevented the Thimerosal-induced depletion in glutathione in neuroblastoma cells (Fig. 5B). In the glioblastoma cells, pretreatment with NAC completely prevented the Thimerosal-induced glutathione depletion and glutathione ethyl ester was partially protective.

DISCUSSION

Considerable concern has been expressed recently over the cumulative dose of mercury given to children through routine immunizations given in the 1990s. The source of mercury in vaccines is the antimicrobial preservative, Thimerosal, containing 49.9% ethyl mercury by weight. All forms of mercury are well known to be neurotoxic, especially during early brain development (Costa et al., 2004). The high affinity binding of mercuric compounds to the thiol (–SH) group of cysteines in essential proteins is thought to be the basis for mercury-induced cytotoxicity. In vivo studies in rodents have shown that ethyl mercury is able to cross the cell membrane and then is converted intracellularly to inorganic mercury which accumulates preferentially in the brain and kidney (Magos et al., 1985). Intracellular accumulation of inorganic mercury was shown to be higher for ethyl compared to methylmercury with equimolar exposure, although the clearance rate of ethylmercury was faster than methylmercury (Magos et al., 1985). A recent in vitro study of Thimerosal exposure to fibroblasts and human cerebral cortical neuron cell lines demonstrated that short term exposure in concentrations similar to those used in the present study induced DNA strand breaks, membrane damage, caspase-3 activation, and cell death (Baskin et al., 2003). The purpose of the present study was to determine whether the mechanism of ethylmercury toxicity was similar to that previously reported for methylmercury. Human glioblastoma and neuroblastoma cell lines were used as surrogates for astrocytes and neurons, respectively, based on a previous study demonstrating similar dose–response profiles to methyl mercury between primary cultures and transformed cell lines (Sanfeliu et al., 2001).

Glutathione provides the major intracellular defense against ROS and oxidative stress-induced cell damage and apoptosis (Meister, 1995). Agents or conditions that deplete mitochondrial glutathione will indirectly increase ROS levels and induce cell death in a variety of cell types (Allen et al., 2001; Marchetti et al., 1997). Mercury and other heavy metals are well known to increase oxidative stress and deplete intracellular glutathione (Naganuma et al., 1990). A major unanswered question is whether mercury-induced depletion of glutathione precedes the increase in ROS or whether mercury-induced ROS induces glutathione depletion. In thymocytes, mitochondrial glutathione depletion was shown to precede the increase in ROS associated with loss of viability and apoptosis (Macho et al., 1997). Whether mercury-induced depletion of glutathione is the initiating factor for increased oxidative stress and cell death in brain cells has not yet been evaluated.

A recent in vitro study of Thimerosal immunotoxicity using immortalized Jurkat T cells demonstrated an

increase in reactive oxygen species and a decrease in intracellular glutathione with increasing concentrations of Thimerosal (Makani et al., 2002). Thimerosal, but not thiosalacylic acid (the non-mercury component of Thimerosal), induced apoptotic cell death in T cells in a concentration-dependent manner as evidenced by mitochondrial release of cytochrome *c*, apoptosis activating factor, and activation of caspases 9 and 3. Exogenous glutathione inhibited activation of these caspases and prevented cell death. These results suggest that, at least in T cells, Thimerosal induces oxidative stress and apoptosis by activating mitochondrial cell death pathways. A subsequent study using cultured human neuron and fibroblast cell lines similarly showed that low micromolar concentrations of Thimerosal induced DNA strand breaks, caspase-3 activation, membrane damage and cell death (Baskin et al., 2003).

In the present study, we evaluated glioblastoma cells and neuroblastoma cells in culture to determine the relative sensitivity of each cell type to Thimerosal-induced oxidative stress and cell death. At equimolar concentrations of Thimerosal, the neurons were found to be much more sensitive to Thimerosal-induced cell death than the astrocytes. In the neuronal cell line, viability was significantly reduced in a concentration-dependent manner at 2.5, 5, 10, and 20 μmol/L Thimerosal after only a 3 h exposure, whereas the astrocytes required a full 48 h exposure for a similar loss of viability (Fig. 3). These results duplicate observations in the same cell lines exposed to similar concentrations of methyl mercury and suggest that the mechanism of ethyl- and methylmercury neurotoxicity is similar. The addition of either *N*-acetylcysteine or gluthatione ethyl ester (100 μmol/L) to the culture medium 45 min before adding 15 μmol/L Thimerosal conferred significant protection against cytotoxicity in both cell lines (Fig. 4). It is likely that the extracellular NAC and glutathione provided partial protection by complexing with the Thimerosal in the culture medium as well as by increasing intracellular glutathione levels. The oxidized form of cysteine (cystine) was protective in astrocytes, but not neurons, consistent with facilitated membrane transport of cystine in astrocytes (Kranich et al., 1998). Neurons depend on glutathione synthesized in the astrocytes and released extracellularly where it is hydrolyzed to cysteinylglycine and cysteine by ectoenzymes to provide neurons with necessary precursors for intracellular gluthathione synthesis (Dringen et al., 1999). In both cell lines, methionine provided no protection against Thimerosal toxicity confirming the inability of either cell type to synthesize cysteine (and glutathione) from methionine.

The intracellular concentration of glutathione before supplementation was 30% lower in neuroblastoma cells compared to the glioblastoma cells. The lower baseline glutathione concentration in the neuronal cell line was associated with increased sensitivity to Thimerosal cytotoxicity (Fig. 5). Thus, sensitivity to Thimerosal was directly proportional to the basal intracellular glutathione concentration. In co-culture studies, astrocytes have been shown to protect neurons against the toxicity of oxidative stress (Dringen et al., 2000a). The provision of glutathione precursors to neurons is a possible explanation for the protective effect of astrocytes. Recent results have confirmed the primary role of astrocytes in glutathione metabolism and antioxidant defense in the brain (Dringen, 2000). Depletion of astrocyte glutathione would therefore indirectly induce oxidative cell death in neurons by depletion of essential glutathione precursors.

In summary, we have shown that human glioblastoma cells are more resistant to Thimerosal cytotoxicity than neuroblastoma cells at doses in the low micromolar range and that the resistance is correlated with higher intracellular levels of intracellular glutathione. The significant protection by NAC and glutathione ethyl ester against Thimerosal cytotoxicity suggests the possibility that supplementation with glutathione precursors might be protective against mercury exposures in vivo. Numerous clinical studies have demonstrated the efficacy of NAC in increasing intracellular glutathione levels and reducing oxidative stress in humans (Anderson and Luo, 1998; Badaloo et al., 2002). Since cytotoxicity with both ethyl- and methylmercury have been shown to be mediated by glutathione depletion, dietary supplements that increase intracellular glutathione could be envisioned as an effective intervention to reduce previous or anticipated exposure to mercury. This approach would be especially valuable in the elderly and in pregnant women before receiving flu vaccinations, in pregnant women receiving Rho D immunoglobulin shots, and individuals who regularly consume mercury-containing fish.

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